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Research Paper

Water-soluble red pigments from *Isaria farinosa* and structural characterization of the main colored component**Palanivel Velmurugan^{*1}, Yong Hoon Lee^{*1}, Kuppanan Nanthakumar², Seralathan Kamala-Kannan¹, Laurent Dufossé³, Sameer AS Mapari⁴ and Byung-Taek Oh¹**¹ Division of Biotechnology, Advanced Institute of Environmental and Bioscience, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan, Jeonbuk, South Korea² Department of Environmental Sciences, Bharthiar university, Coimbatore, Tamil Nadu, India³ Université de la Réunion, LCSNSA (Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments), ESIROI-IDAI (Ecole Supérieure d'Ingénieurs Réunion Océan Indien, Département Innovation et Développement Agroalimentaire Intégré), Parc Technologique, Sainte-Clotilde, La Réunion, France⁴ Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark (DTU), Kgs Lyngby, Denmark

The present study describes the red pigment synthesized by the filamentous fungi *Isaria farinosa* under submerged culture conditions. The pigment production was optimal under the following conditions: pH 5, agitation speed 150 rpm, temperature 27 °C, incubation time 192 h, light source total darkness, sucrose and glucose as carbon source, yeast extract, meat peptone and monosodium glutamate at a fixed concentration of 3% as nitrogen source. The addition of 10 mM CaCl₂ to the culture medium increased the biomass and pigment production. Structural elucidation of the pigment using gas chromatography-mass spectrometry, Fourier transform infrared spectroscopy and ¹H nuclear magnetic resonance spectroscopy revealed that the red pigment contains an anthraquinone-related compound. In addition, the isolated pigment was water soluble, and was stable when exposed to salt solution (96.1% of stability after treatment with sodium chloride), acid (72.1% with citric acid), heat (86.2% at 60 °C), and sunlight (99.4%). These results are promising to further exploit the fungal culture of *Isaria farinosa* for producing the red pigment and, subsequently, to considerably increase its yield. The study has commercial importance in the production of *Isaria farinosa* pigment for industrial application after considerable toxicological examination.

Keywords: Anthraquinone / Isolation / *Isaria farinosa* / Optimization / Pigment / Structure

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Introduction

Increased use of synthetic dyes and chemicals in the textile, foodstuff, cosmetic and pharmaceutical industries have resulted in the generation of large quantities of effluent that contain high levels of dyes and toxic materials. The presence of these pollutants in the efflu-

ents may pose environmental disposal problems due to their non-degradable and persistent nature. Moreover, the presumed or actual noxious effects of some synthetic dyes on human health, such as skin cancer and allergic reactions, have raised a great deal of concern [1]. Therefore, the environmentally hazardous synthetic dyes must be replaced with cost-effective natural and environment-friendly dyes or pigments.

Numerous studies have reported the application of plant-based pigments in dyeing processes [2, 3]. Even though a number of plant pigments were isolated and characterized, only a few are available in sufficient quantities to be used in the dyeing industries [4]. Hence, it is suggested to exploit the potential of other

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biological sources such as fungi (both moulds and yeasts), bacteria, and algae, with appropriate selection, mutation or genetic engineering techniques, to improve the pigment production yield compared to the wild-type organisms [5, 6].

The microbial production of pigments [7–10] with different shades such as yellow, orange, pink, reddish brown and red has been reported. Molecules such as quinones (anthraquinones and naphthaquinones), dihydroxy naphthalene melanin (a complex aggregate of polyketides) [11], and flavin compounds (riboflavin) have been well characterized from different fungi [12]. Among the different shades, red pigment is widely used in the dyeing industries. The red-colored pigments are isolated from several fungi and have been extensively studied [5–8, 13]. Santis *et al.* [1] extracted the red pigment from *Monascus purpureus* and used it for wool dyeing. Similarly, Nagia and El-Mohamedy [14] assessed the dyeing potential of red pigment produced by *Fusarium oxysporum* for woolen materials. Cho *et al.* [15, 16] reported a red pigment of unknown structure to be produced by *Paecilomyces sinclairii*. However, there is no report about *Isaria farinosa* (synonym of *Paecilomyces farinosus*) red pigment and its structure elucidation. Moreover, numerous studies indicated that pigment production in submerged culture is affected by various environmental factors, particularly the pH of the medium, temperature, agitation, and carbon and nitrogen sources [17, 18]. Therefore, we studied the effects of several environmental parameters on red pigment production by *I. farinosa*.

Hence, the present study focuses on the optimal red pigment production by *I. farinosa* in synthetic liquid medium. The structure of the red pigment was also elucidated and the potential of the pigment for industrial application was investigated by studying the physiochemical parameters of the pigment.

Materials and methods

Study area and samples

Soil samples were collected from Nilgris, a high-altitude site and biodiversity hotspot in the Western Ghats of the state of Tamil Nadu, India [19]. A total of 51 soil samples were collected from diverse locations, transported on ice to the laboratory and processed within 24 h. Pigment-producing fungi were isolated by serially diluting 1 g of the soil sample in sterile distilled water, and 1 ml of the appropriate dilutions was plated by pour plate technique on potato dextrose agar (PDA) (HiMedia, Mumbai, India). Later, the plates were incu-

bated (27 °C for 10 d) and observed for fungal growth. Pigment-producing fungi were picked, purified and stored in PDA at 4 °C for further studies. Among the isolates, *I. farinosa* was selected based on its ability to produce red pigment. The isolate was identified based on morphological and cultural criteria by the commercial Fungal Identification Service from the Agharkar Research Institute, Pune, India.

Optimization of culture conditions

The isolated and identified *I. farinosa* strain was cultivated individually in E-flasks on 100 mg/l of defined medium containing (per liter of deionized water): 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1.4 g K_2HPO_4 , 0.6 g KH_2PO_4 , 0.8 mg $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.8 mg $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 0.8 mg $\text{NaMoO}_4 \cdot 2 \text{H}_2\text{O}$, 0.4 mg $\text{MnSO}_4 \cdot 2 \text{H}_2\text{O}$, and 0.2 mg $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$. The optimum growth conditions such as pH, temperature, agitation time, calcium chloride (CaCl_2), light, and carbon and nitrogen sources were determined according to standard methods [15]. Experiments were conducted in shake flasks; fungal growth and pigment production were monitored for 7 d. All experiments were performed in duplicates and repeated twice. Parameters such as pH, agitation, temperature, and carbon and nitrogen source were adjusted to prepare optimized media. Different carbon and nitrogen sources were added at 3% (w/v) to meet the required carbon and nitrogen content. To study the effect of different wavelengths of light on the growth and pigment production, the experiment was set up based on the principle that a colored glass paper allows only its particular color of light to pass through, as it filters out the other colors of the spectrum [10]. Fermentation flasks were wrapped in colored glass papers of red, blue and green and placed at equal distance (20 cm) from a light source (Philips T8, Master TL D, T8 51, 840 W; with wavelengths of 492–455 nm for blue, 577–492 nm for green, 597–577 nm for yellow, and 780–622 nm for red). To study the effect of direct illumination, the flasks were directly kept under the light source, and to study the effect of total darkness, the flasks were covered with black paper. The setup was kept as given below (20 cm) from a light source inside the incubator installed with light. Each flask inoculated with *I. farinosa* was incubated at 27 °C for 7 d.

Extracellular and intracellular red pigment estimation

To estimate the extracellular red pigment, the entire culture broth (approximately 50 ml) was extracted with a mixture of ethanol and water (1:1, v/v) for 1 h on a rotary shaker (100 rpm) at room temperature and filtered through a pre-weighed Whatman Glass Filter

Binder Free Grade (GF/C) disc. To determine the mycelial dry weight, the disc was washed with 5–10 ml of the same mixture and dried for 10 h at 105 °C. To estimate the intracellular red pigment, the mycelia were washed in copious amounts of deionized water several times; 1 g of washed mycelia was taken in a conical flask; 20 ml of 95% ethanol was added and kept in a hot water bath under continuous agitation for 12 h. The lid of the flask was closed with aluminum foil to prevent ethanol evaporation. Both extracellular and intracellular filtrate was separately made up to 100 ml using the extraction mixture and, if necessary, the pigment was diluted with the same mixture; the absorbance was measured at 400, 470 and 500 nm. These wavelengths represent absorption maxima for yellow (400 nm), orange (470 nm) and red (500 nm) pigments [20]. Pigment production (units of absorbance, UA) was calculated by multiplying the absorbance values by the dilution factor [21]. The results for red pigment production were expressed as the absorbance units (U) multiplied by a dilution factor according to Lee *et al.* [22].

Preparation of *I. farinosa* red pigment for characterization

Red pigment was extracted from the culture filtrate by a filtration membrane (80 holes/cm²), and the filtrate was lyophilized using a Buechi rotary evaporator at 45 °C. Red pigments were extracted from the lyophilized powder (crude extract) with hexane, ethyl acetate, and methanol in sequence [23]. The extracted pigments were further characterized by the following techniques.

Pigment characterization

Ultraviolet-visible spectrophotometry: The maximum absorbance of extracted and dried red pigment powder was determined by spectrophotometer (Hitachi-3210 UV-Vis) at 500 nm wavelength.

Gas chromatography-mass spectrometry: The main peak of red pigment was chosen and its molecular weight was determined by direct injection of pure molecule into the gas chromatography-mass spectrometry (GC-MS) system (GC 8000 Top MD 800 MS, Thermo Fisher Instruments, GC fission MS) which was coupled with an AB Ms35 column 30 m × 0.25 mm × 0.41 mm. Helium was used as the carrier gas and the oven temperature was held at 100 °C for 1 min and then increased at a gradient of 10 K/min to the final temperature of 250 °C [24].

Fourier transform infrared spectroscopy: The dried crystals of the red pigment were scanned by a Shimadzu FT-IR 8000 spectrophotometer in the 4000–400 cm⁻¹ range using the KBr method at 27 °C.

¹H nuclear magnetic resonance spectroscopy: The purified pigment was dissolved with ethyl acetate and the sample was injected into a nuclear magnetic resonance (NMR) spectrometer (Varian Mercury V × 300; Varian, USA) with the following conditions: pulse sequences, 2 pul; solvent, D₂O; ambient temperature; mercury-300BB"nk300"; relax delay, 1.000 s; pulse, 45.0°; acquisition time, 1.997 s; width, 4803.1 Hz; repetitions, 16; observed H, 300.0771372 MHz; data processing line broadening, 0.2 Hz; FT size, 32768; total time, 53 s.

Stability test for red pigment: Stability testing of the pigment was carried out according to the method reported by Srivastava *et al.* [25] and Perumal *et al.* [26]. Of the fungal pigment extract, 10 ml was taken in a test tube and incubated in a water bath set at 40, 50, 60, 70, 80, 90, and 100 °C for 10 min. After incubation, it was cooled and subjected to analysis using an ultraviolet-visible (UV-vis) spectrophotometer (500 nm). Another set of test tubes each containing 10 ml of the fungal pigment extract was adjusted to pH values of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0, and mixed thoroughly, incubated for 10 min and measured using a UV-vis spectrophotometer. Similarly, the test tubes containing 10 ml of the fungal pigment extract were incubated under different stress conditions, such as in a hot-air oven at 60 °C overnight (12 h), sunlight for 2 h, and steam at 121 lbs pressure for 20 min, and then subjected to UV-vis spectrophotometer analysis [26]. Another set of test tubes each containing 10 ml of the fungal pigment extract were incubated under a UV light source for 12 h. Salt solution (sodium chloride and aluminum chloride) with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1% was added to the test tubes containing 10 ml pigment extract and kept in a warm water bath for 1 h to test its stability against dyeing for its use in textile application. Acid solution (nitric acid and citric acid) with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1% was added to 10 ml each of the fungal pigment extracts and kept at room temperature, before subjecting to UV-vis spectrophotometer scanning analysis.

The percentage of stability (%E) was calculated using the equation

$$\%E = [(A_0 - A_1) \times 100] / 10 \quad (1)$$

where A_0 stands for the absorbance of the pigment before treatment and A_1 stands for the absorbance of the pigment after treatment at λ_{\max} of the pigment used.

Results and discussion

The isolated pigment showed unique light absorption at 500 nm, indicating red pigment (Fig. 1). In addition,

the color of the isolated pigment changed according to variations in pH of the aqueous solution. As the pH of the aqueous solution shifted to alkaline, the maximum absorption wavelength increased, associated with a variation in color in the following manner: acidic solution, yellow (570–580 nm), red (500–507 nm); neutral solution, violet (529–536 nm); and alkaline solution,

pale violet (540–580 nm) (data not shown). Cho *et al.* [15] also observed a similar variation in the color of red pigment isolated from *P. sinclairii*. This infers that the pigments produced by *I. farinosa* and *P. sinclairii* are of a similar chemical nature, and confirms the relatedness of the two species as the genera appear to be the same except for the nomenclature system adopted.

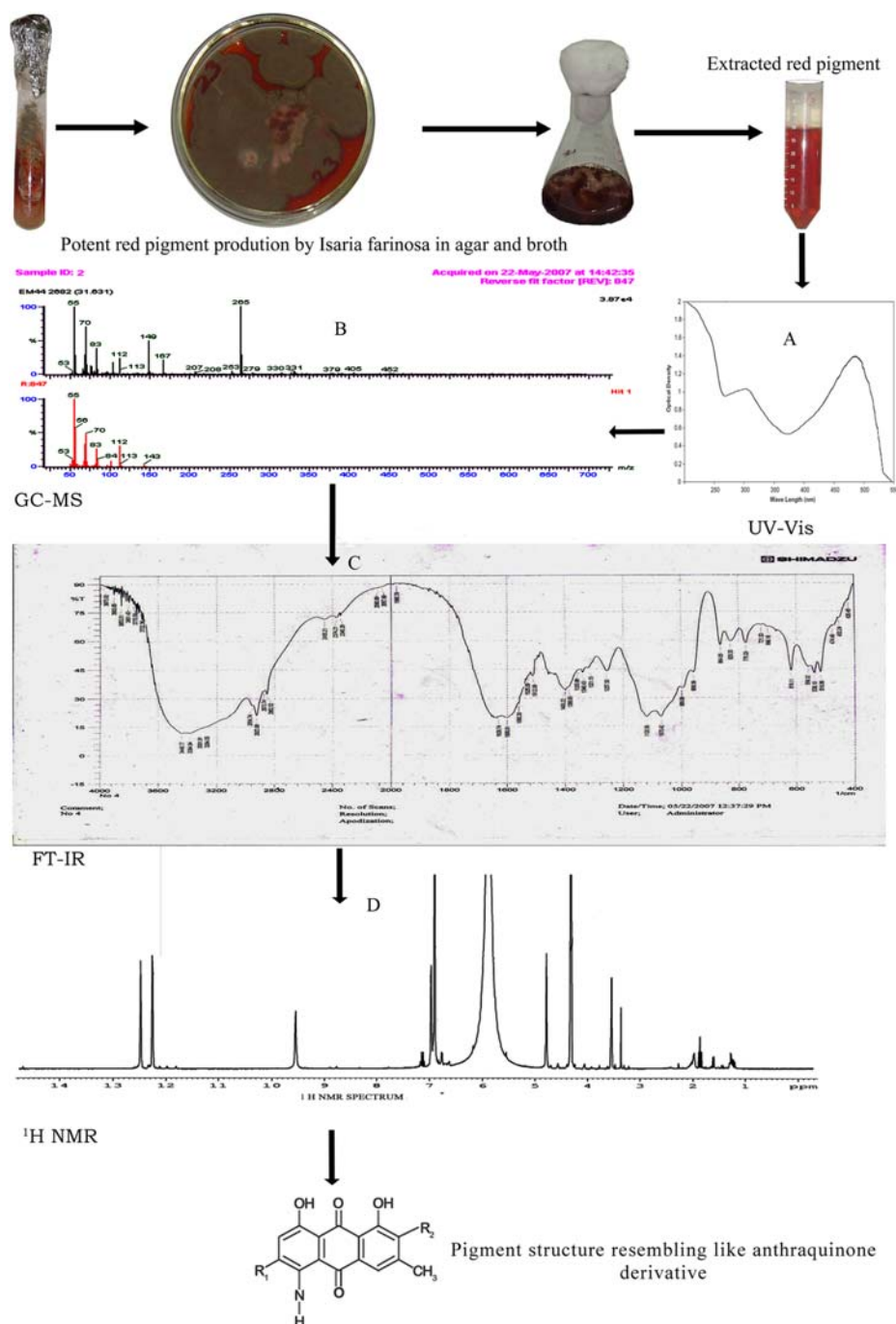


Figure 1. Cultivation of *I. farinosa*, extraction of red pigments, structure prediction of the main colored component by UV, GC-MS, FT-IR, NMR spectra, and its predicted structure.

Effect of pH and temperature on red pigment production

The pH of the culture medium seems to be a crucial factor in determining the fungal growth and subsequent pigment production. Numerous studies have reported that most of the filamentous fungi need an acidic pH (5.0–6.0) as optimum for growth and pigment production in submerged culture [15, 27]. Hence, it is to investigate the effect of pH on biomass and extracellular and intracellular red pigment production. *I. farinosa* was cultivated at different pH values (4.0–9.0) and the results are presented in Fig. 2a. Maximum production of extracellular pigment (23 U), intracellular pigment (9 U), and biomass (3 g/l) was observed at pH 5, and minimum production was observed at pH 8 and 9. The increased pigment production under acidic conditions could be due to the formation of less biomass, suggesting that the pH of the medium might affect the transport of certain media constituents such as glucose and nitrogen sources [22]. The results are consistent with a previous study conducted by Carels and Shepherd [18], where increased pigment production was observed under acidic conditions in a glucose-salts medium. Generally, a dramatic pH drop leads to the impairment of pigment–amine interactions [28]. Temperature is another important factor as it influences the metabolic activity of microorganisms and, subsequently, their growth. To test the optimal temperature for biomass and pigment production, *I. farinosa* was cultivated at various temperatures (20–37 °C). Consequently, the optimal temperature for both biomass and pigment production was found to be 27 °C (extracellular 16.1 U, intracellular 9.6 U, biomass 4 g/l), which clearly indicated the mesophilic nature of the fungus (Fig. 2b). Taking into account that higher fungi usually require longer periods for submerged culture, thereby exposing them to the risk of contamination, this optimal temperature is regarded as a favorable physiological trait of *I. farinosa*. This observation is in agreement with Cho *et al.* [15]. Sardaryan *et al.* [29] reported that an optimum temperature between 27 and 30 °C is required for the growth of and pigment production in *P. sinclairii* and *Penicillium oxalicum*. Hence pH 5 and a temperature of 27 °C were used as optimum conditions for further studies.

Effect of agitation speed and incubation period on red pigment production

The biomass and pigment production (extracellular and intracellular) at various agitation speeds (100–250 rpm) are presented in Fig. 2c. The amount of pigment produced varied with the agitation speed. Both the pig-

ment and biomass production increased up to 150 rpm (extracellular 26.4 U, intracellular 19.9 U, biomass 6 g/l) and decreased thereafter. According to Lee *et al.* [22], in submerged mold culture, mycelia may grow into homogeneous and filamentous suspensions, or show pellet type growth or the intermediate forms between pulp and pellet type. Cultivation at optimal agitation speed produces mycelial forms of intermediate type and provides an effective means of increasing the pigment yield. At 150 rpm, the cells showed intermediate forms between the pulp and pellet type, and the pigment production was better. At an agitation speed of 250 rpm, the pellet-type mycelia predominated and pigment production drastically decreased. Hajjaj *et al.* [30] have shown the strong influence of agitation on the growth and secondary metabolite production in higher fungi.

The biomass and pigment production rates at different incubation periods were analyzed and the results are presented in Fig. 2d. Maximum biomass (5.1 g/l) and extracellular pigment (19.2 U) production were obtained after 192 h of incubation, followed by intracellular red pigment production at 96 h (11.0 U), with a slight reduction (10.5 U) at 192 h due to pH change. From the results, it is evident that a lag phase occurred during the first 48–96 h. Similarly, the incubation period from 192 to 384 h showed a stable biomass and pigment production. Earlier reports demonstrate that both liquid- and solid-state fermentation need longer time to obtain high biomass, pigment and metabolite production [9, 16]. Based on the results, the following set of conditions was found to be optimal for maximal biomass and pigment production: pH 5.0, temperature 27 °C, agitation at 150 rpm, and incubation time 192 h.

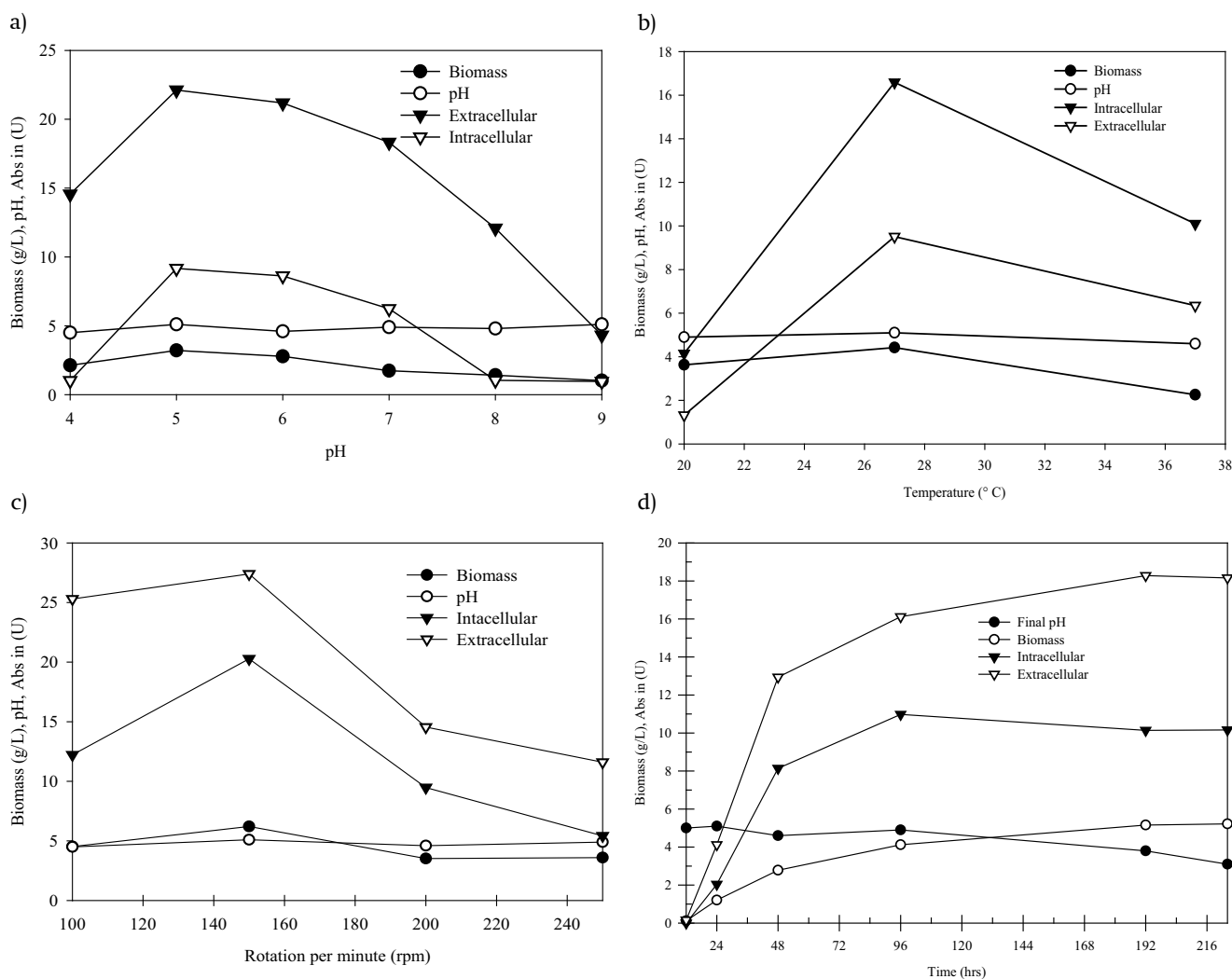
Effect of bio-element CaCl₂ and different light sources on red pigment production

Bio-elements are also important factors affecting pigment production in several microorganisms [31]. The presence of CaCl₂ in the medium can enhance the mycelial growth and pigment production in *P. sinclairii*, as reported by Cho *et al.* [15]. Hence, we intended to see the influence of CaCl₂ in the medium on biomass and pigment production. The isolate was cultivated in the above-mentioned optimized culture medium with 5–11 mM CaCl₂, and the results are presented in Fig. 2e. Maximum pigment production (extracellular 22.8 U, intracellular 10.9 U, biomass 3.5 g/l) was achieved at a concentration of 10 mM CaCl₂ (Fig. 2e). It is clearly observed from Fig. 2e that both intracellular and extracellular pigment production increased with

the increase in element concentration from 7 to 9 mM CaCl_2 , and maximum production was at 10 mM CaCl_2 . However, a marked decrease in the pigment and biomass production was observed at a concentration of 11 mM CaCl_2 . The decreased biomass and pigment production could be due to an inhibitory effect of CaCl_2 . The results are in agreement with Roisin *et al.* [32], where a decreased production of red pigments in *Gibberella fujikuroi* was observed in the presence of a high CaCl_2 concentration.

In the fungal kingdom, light also plays an important role; it can regulate growth and its direction, sexual and asexual reproduction, and pigment production, all of which are important aspects for the survival and dissemination of the fungal species [10]. In this study, the absorption spectra of the pigments extracted from light- and dark-grown *I. farinosa* (Fig. 2f) indicated that the pigment composition largely changes depending on

the light conditions. Incubation in total darkness resulted in increased biomass and pigment production (about twofold), followed by blue and red wavelengths. There was no interesting increase in biomass and pigment production at white unscreened, green, and yellow wavelengths. However, blue wavelengths led to a reduction in biomass. The effects of white unscreened, green, and yellow wavelengths on metabolism, growth, sexual and asexual development, pigment formation, tropism, and other phenomena have been studied in a wide variety of fungus. The effectiveness of white unscreened, green, and yellow wavelengths in inhibiting pigment production was not surprising. The vast majority of photoresponses, from growth responses to phototropism, studied in fungi are mediated by the photoreceptors that absorb white unscreened, green, and yellow wavelengths.



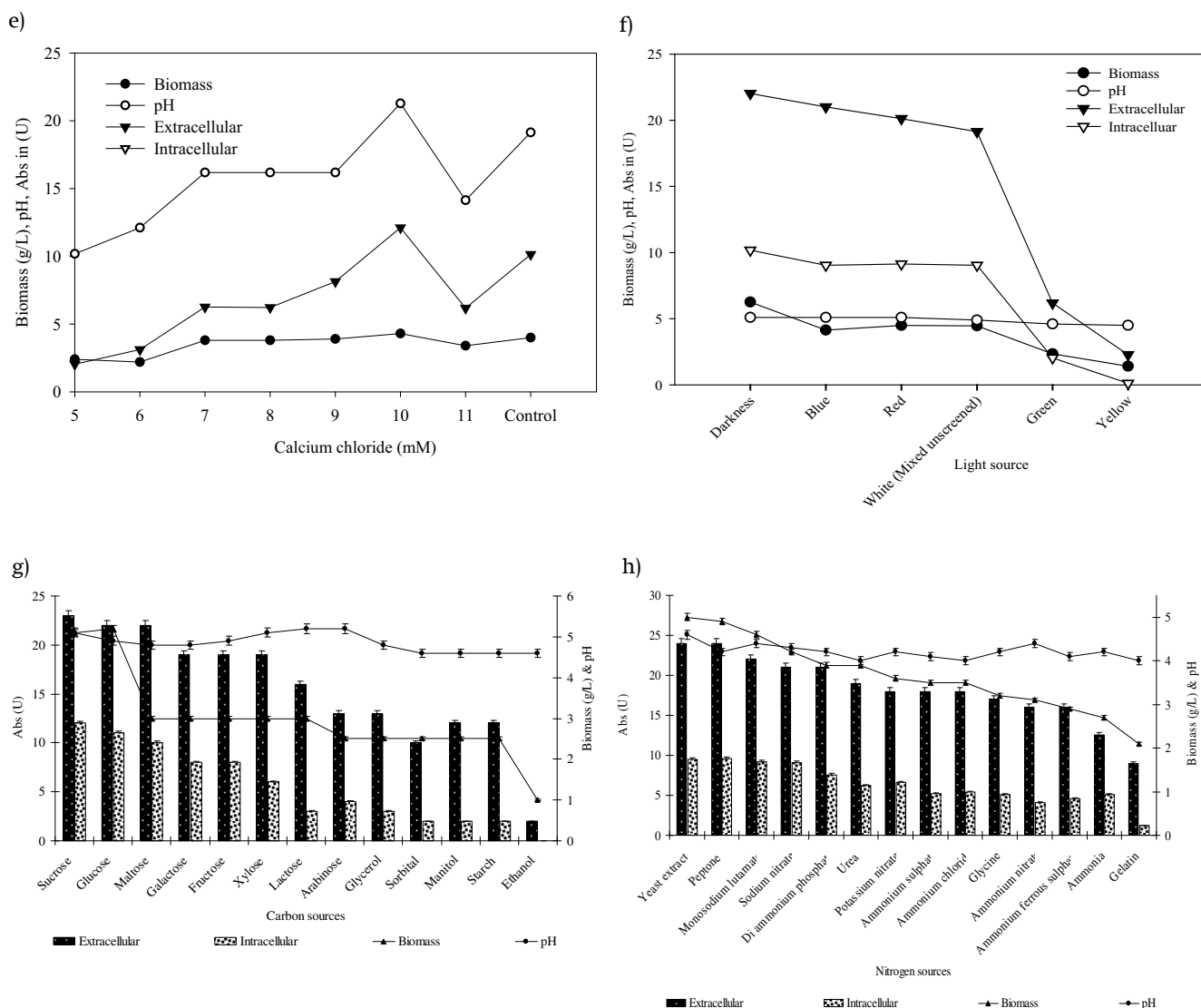


Figure 2. Effects of (a) initial pH, (b) temperature, (c) agitation, (d) production time, (e) calcium chloride, (f) light frequency, (g) carbon source, and (h) organic and inorganic nitrogen sources on the biomass and intra- and extracellular pigment production of *I. farinosa*.

Effect of different carbon and nitrogen sources on red pigment production

To select a suitable carbon source for biomass and pigment production, *I. farinosa* was cultivated in culture media containing various carbon sources at 3% (w/v). Of the 13 carbon sources examined, glucose, sucrose, mannitol, and maltose were relatively encouraging for the biomass and pigment production. Maximum biomass (7 g/l) was achieved in sucrose and glucose medium while maximum pigment production (extracellular 23 U, intracellular 12 U) was obtained in soluble sucrose (Fig. 2g). This result is in agreement with Cho *et al.* [15] who reported red pigment production by *P. sinclairii* in submerged culture conditions. Lee

et al. [22] confirmed that glucose is a superior substrate for pigment production by *Monascus* spp.

To study the effect of nitrogen sources on pigment production, 14 organic and inorganic nitrogen sources were used at a fixed concentration of 3% (w/v). Organic nitrogen sources gave rise to high biomass and pigment production when compared with inorganic nitrogen sources. Yeast extract, meat peptone, and monosodium glutamate had a positive effect on biomass and pigment production, whereas gelatin strongly inhibited red pigment synthesis and biomass production. Among all the nitrogen sources tested, meat peptone gave the highest yield for red pigment production (Fig. 2h). It has been reported that various types of peptone supported greater pigment production in various pigment-producing

fungi [16]. Among the inorganic nitrogen sources, sodium nitrate and diammonium phosphate gave a considerable raise in pigment and biomass yield, whereas with ammonia there were much lower biomass and pigment yields. It has been reported that various kinds of amino acids are essential for secondary metabolite biosynthesis [18].

Elucidation of red pigment structure

UV-vis spectra of the new *I. farinosa* red pigment: The red pigment was subjected to extraction using ethanol [23]. The absorbance of the new *I. farinosa* red pigment is illustrated in Fig. 1A. The maximum absorption wavelength was observed at 500 nm, which is similar to that reported in the literature for *Monascus* and *P. sinclairii* red pigment [16, 23, 33].

GC-MS: GC-MS of the *I. farinosa* red pigment showed a large peak at m/z 265 (Fig. 1B) that is consistent with the $(M + H)^+$ ion of the compound.

Fourier transform infrared: The infrared spectrum of the *I. farinosa* red pigment is presented in Fig. 1C. The Fourier transform infrared (FT-IR) spectrum was recorded on a Shimadzu IFS 66V FTIR. The main absorbance peaks included 3440.07, 3301.09, 2923.08, 2852.05, 2455.02, 2374.02, 3345.02, 1629.07, 16008.08, 1400.02, 1388.06, 1340.02, 1257.05, 1220.05, 1120.05, 1070.08, 864.00, 829.03, 775.03, 619.01, 538.01, and 516.08 cm^{-1} . The peaks at 3440.07, 1220.05, and 1070.04 cm^{-1} suggest that there could be a hydroxide bond in the molecules. The peaks at 2374.02 and 1388.06 cm^{-1} indicated that there might be NH groups. The peak at 2923.08 cm^{-1} was very sharp, indicating that there were multiple CH_2 groups. The peaks at 1629.07, 16008.08 and 1400.02 cm^{-1} indicate the presence of a benzol structure and a CH_3 group.

NMR: Assignments of the ^1H NMR resonances of the *I. farinosa* red pigment were obtained by using Varian spectra (Fig. 1D) recorded with a mixing time of 53 s in order to detect direct and relay through-bond connections. The ^1H NMR spectrum showed: a three-proton singlet at δ 3.324, a keto group adjacent to an amino group; a three-proton singlet at 4.52; a methane group; two one-proton doublets at δ 4.52 and 4.89, with coupling constants characteristic of a disubstituted A ring; two one-proton singlets at δ 9.56, indicating another disubstituted aromatic ring; chelated hydroxyls were also seen at δ 12.32 and 12.71, indicating an aromatic ring and one chelated hydroxyl group at the end point. Fig. 1 shows the chemical structure of the *I. farinosa* red pigment. Totally, it consists of 2-hydroxy, 1-methoxy amino group with hydrophilic side chain. The amino group linked to the anthraquinone may be primary,

with R_1 and R_2 being H. Alternatively, it may be secondary, with R_1 being H and R_2 being a C_{1-4} lower alkyl. Alternatively, it may be tertiary, with R_1 and R_2 being C_{1-4} lower alkyls. For example, R_1 and R_2 may be CH_3 . Alternatively, R_1 and/or R_2 may be based on an alkyl chain but having additional functionality. For example, R_1 and/or R_2 may have the formula $\text{CH}_2\text{CH}_2\text{OH}$. Some metabolites have been described in genetically related strains belonging to the *Paecilomyces* or *Isaria* genera. α -Pyrones and cyclohexenones were isolated from *Paecilomyces lilacinus*, and their structures were characterized [34]. Petersen *et al.* [35] reported the pigments paecilquinones A–F from *Paecilomyces carneus* P-177. Similarly, Cheng *et al.* [36, 37] reported two new yellow pigments from *P. farinosus*. However, in our study, we identify the red pigment produced and the main colored compound as anthraquinone.

Stability of pigment

The pigment of the fungi was subjected to various physical and chemical treatments to test its stability and the results are presented in (Table 1). The solubility of the pigment in water was excellent and the color content was read at 500 nm. The pigment was somewhat hygroscopic in nature and the hue test resulted as red color. The initial color of this pigment was red (pH 5.0), and different shades of colors resulted when the pigment extract was adjusted to pH values of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0. At pH 2.0, 8.0, 10.0, 12.0 and 14.0, the pigment lost its original red color and turned white by denaturation. At all other pH values, different shades of color such as brown (pH 4.0), yellow (pH 5.0), brownish orange (pH 8.0 and 10.0), and pale red (pH 12.0) were observed (Table 1). Precipitation was observed at the bottom of the tube containing the pigment at pH values of 2.0, 12.0, and 14.0. The pigment stability was tested at different temperatures ranging from 40 to 100 °C, and the pigment was shown to be stable at 60 °C and below. The pigment subjected to steaming and sunlight exposure showed no change of color. The pigment was subjected to various salt treatments and was stable at 0.1, 0.2, and 0.3% sodium chloride (96.1) and in ammonium chloride (90.2). During acid treatment, 0.1 and 0.2% nitric acid (58.4) and citric acid (72.1), it showed an increase in stability. The fungal pigment from *I. farinosa* was stable under sunlight, at various temperatures and also during various treatments. The results of the present study indicate that the red pigment produced by *I. farinosa* can be used as a textile colorant. Interestingly, the OH group will facilitate the interaction between textiles and the pigment when dyeing with an appropriate mordant.

Table 1. Properties of *Isaria farinosa* red pigment and its stability after different treatments.

Parameter	Properties	
Water solubility	soluble	
Color content, absorption [nm]	500	
Hygroscoy	little	
Hue	dark red	
Parameter	Stable in	Stability [%]
pH, 2–14, 10 min	pH 4, 5, 6, and 7	99.0
Dry heat, 60 °C for 12 h	–	86.2
121 °C for 20 min	–	95.9
UV light for 12 h	–	99.2
Sunlight for 2 h	–	99.4
Sodium chloride (0.1–1% w/v), pH 7.0, 1 h	0.1, 0.2, 0.3, 0.4, 0.5, and 0.6%	96.1
Aluminum chloride (0.1–1% w/v), pH 7.0, 1 h	0.1, 0.2, and 0.3%	90.2
Nitric acid (0.1–1% w/v), pH 7.0, 1 h	0.1 and 0.2%	58.4
Citric acid (0.1–1% w/v), pH 7.0, 1 h	0.1 and 0.2%	72.1

Conclusions

The most significant outcome of this study was the increased yield of red pigment from *I. farinosa* by culturing the fungi under various nutritional conditions. It could be shown that *I. farinosa* responded by high pigment production while using various parameters. The results of the optimization, stability and biochemical characterization indicate that the isolated pigment could be an alternative for commercial dyes used in textiles. Moreover, the structural elucidation also showed that structure of the main pigment constituent was closely related to the anthraquinone structure. In this regard, further compound characterization and critical toxicity evaluation with animal models is essential. This may reveal novel sources of such anthraquinone-based dyes from the relatively unexplored fungal biodiversity. To the best of our knowledge, this is the first study to report red pigment production by *I. farinosa*.

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